Eicosanoids Mediate Nodulation Responses to Bacterial Infections in Larvae of the Tenebrionid Beetle, *Zophobas atratus*

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Nodulation is the temporally and quantitatively predominant cellular defense response to bacterial infection in insects and other invertebrates. Inhibition of eicosanoid biosynthesis in larvae of the tenebrionid beetle, *Zophobas atratus*, immediately prior to intrahemocoelic injections with heat killed preparations of the bacterium, *Serratia marcescens*, strongly reduced the nodulation response. Separate treatments with specific inhibitors of phospholipase A_2, cyclooxygenase, and lipoxygenase reduced nodulation, supporting the view that nodule formation is a complex process involving both cyclooxygenase and lipoxygenase products. The inhibitory effects of the phospholipase A_2 inhibitor, dexamethasone, on nodulation were apparent 1 h after infection, and the effects increased, relative to controls, over 24 h. The dexamethasone effects were expressed in a dose-dependent manner, and they were reversed by treating bacteria injected insects with the eicosanoid-precursor polyunsaturated fatty acid, arachidonic acid (C20:4n-6). Treatments with the saturated fatty acid, 16:0, which is not an eicosanoid precursor, did not reverse the dexamethasone effects on nodulation. The insects contain low levels of three eicosanoid precursor polyunsaturated fatty acids in six different tissues, and fat body preparations are competent to produce both cyclooxygenase and lipoxygenase products. These findings strongly support the identification of nodulation as a specific insect cellular defense mechanism that is mediated by eicosanoids.

Immunity   Nodulation   Eicosanoids   *Zophobas atratus*   Phospholipase A_2

INTRODUCTION

Insects elaborate two broad categories of defense responses to bacterial infections: humoral and hemocytic (Gupta, 1986; Gupta, 1991; Boman and Hultmark, 1987; Bulet *et al.*, 1991). Humoral responses require several hours for their full expression, and involve induced synthesis of anti-bacterial proteins such as cecropins (4 kDa), attacins (12–23 kDa), diptericins (8 kDa) and defensins (4 kDa) (Boman and Hultmark, 1987). The detergent properties of these anti-bacterial proteins disrupt bacterial cell membranes. Insects also synthesize lysozymes, enzymes that directly attack bacteria by hydrolyzing their peptidoglycan cell walls (Dunn, 1986). Hemocytic responses feature direct cellular interactions

between circulating hemocytes and bacteria, and these typically occur within minutes after infections, rather than hours. Specific cellular defense mechanisms include phagocytosis, nodulation, and encapsulation (Gupta, 1986; Gupta, 1991).

We recently suggested that insect cellular immune responses to bacterial infections are mediated by eicosanoids (Stanley-Samuelson *et al.*, 1991). We observed the effects of pharmaceutical inhibitors of eicosanoid biosynthesis on the ability of tobacco hornworms, *Manduca sexta*, to clear artificial infections from their circulation. Inhibition of total eicosanoid biosynthesis, with injections of the phospholipase A_2 (PLA$_2$) inhibitor dexamethasone, severely reduced the hornworms' ability to clear infected bacteria from their hemolymph, and increased larval mortality due to the bacterial infections. Inhibition of either of two specific eicosanoid biosynthesis pathways, cyclooxygenase or lipoxygenase, also reduced clearance of bacteria, but to a lesser extent compared to the effects of completely inhibiting eicosanoid biosynthesis. On the basis of these findings, we proposed

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that eicosanoid products of the cyclooxygenase and lipoxygenase pathways are involved in insect immune responses to bacterial infections (Stanley-Samuelson et al., 1991). Because most of our experiments were done during the first hour post infection (PI), long before the appearance of anti-bacterial proteins in insect hemolymph, we suggested that eicosanoids mediate one or more hemocytic defense responses (Stanley-Samuelson et al., 1991).

Because nodule formation is one of the generalized cellular responses to bacterial infections that is observed in many, if not all, insect species (Gupta, 1991), and because nodulation (Fig. 1) is quantitatively the most important cellular defense response to bacterial infections (Horohov and Dunn, 1983), we investigated the idea that nodulation is mediated by eicosanoids in tobacco hornworm larvae. Larvae that were treated with dexamethasone before injecting bacteria into their hemocoel formed far fewer nodules at 6 h PI, compared to control insects. The dexamethasone effects could be reversed by treating the infected insects with the eicosanoid-precursor fatty acid 20:4n-6, and we concluded that nodulation is one of the cellular immune responses to bacterial infections that is mediated by eicosanoids (Miller et al., 1994).

On the basis of our findings with Manduca (Miller et al., 1994), we predicted that eicosanoids will prove to be crucial mediators of nodulation in arthropods. Here, we describe the outcomes of experiments that were designed to test this hypothesis. We report that treating larvae of the tenebrionid beetle, Zophobas atratus, with eicosanoid biosynthesis inhibitors prior to injecting them with heat killed bacteria resulted in reduced nodule formation, and that both cyclooxygenase and lipoxygenase products seem to be involved. As in the experiments with Manduca, the effects of eicosanoid biosynthesis inhibition were reversed by treating the beetle larvae with 20:4n-6. These findings strengthen our assertion that eicosanoids mediate nodule formation in a phylogenetically wide range of arthropods, perhaps all of them.

MATERIALS AND METHODS

Organisms

Late-instar larvae of the beetle Zophobas atratus, weighing at least 1.0 g, were used in all experiments. Larvae were purchased from a local pet store, and maintained on a laboratory bench in terraria containing wheat bran and oatmeal. Water was provided by keeping damp towels on top of the bran. The larvae were used for experiments within 10 days of purchase. Cultures of a non-pigmented strain of Serratia marcescens and nutrient broth (Difco) were purchased from Carolina Biological Supply (Burlington, NC). Bacteria were grown in 50 ml of nutrient broth in an environmental shaker at 37°C and 100 r.p.m. Bacteria were grown to a titre of 10^7 cfu per ml, then heat killed in an autoclave. The heat killed bacteria were concentrated and kept under sterile conditions at 4°C.

Injections and assays for nodulation

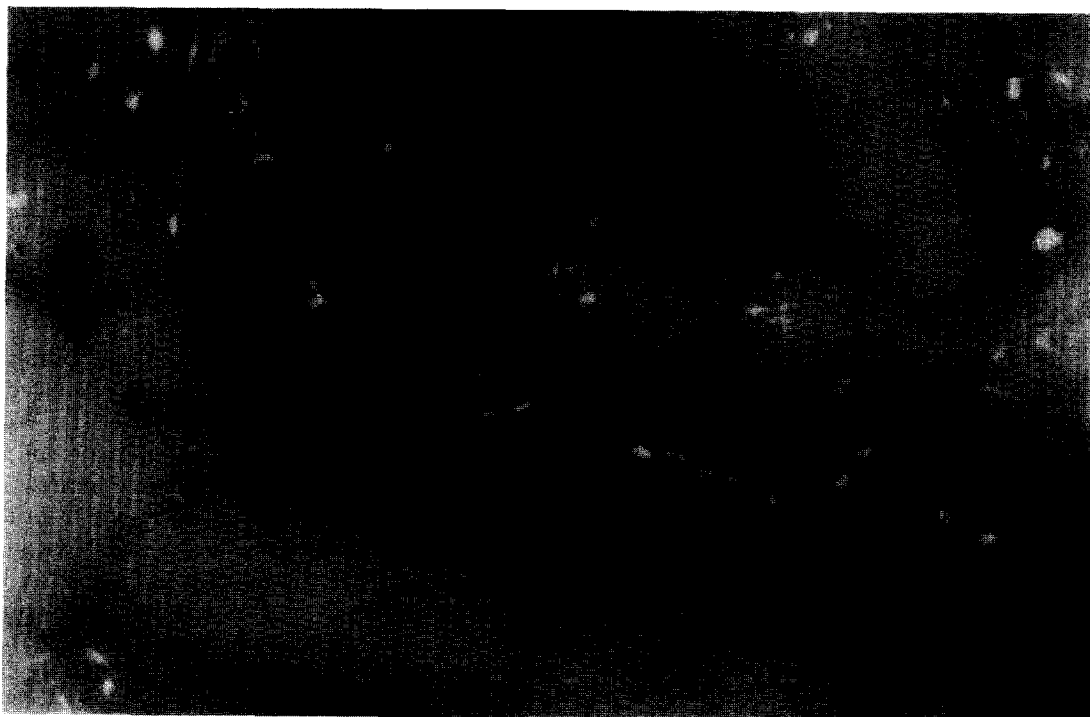Test larvae were injected with either the PLA_2 inhibitor dexamethasone [(11ß,16α)-9-fluoro-11,17,21-trihyd-

FIGURE 1. A microphotograph (total magnification, 60×) of melanized nodules in response to bacterial infection. The solid arrow points to one of several visible nodules, seen in situ against the background of larval fat body. This photograph was taken during routine assessment of nodulation at 18 h PI using an Olympus SZ series stereomicroscope equipped with a SC 35 camera. Photograph by J. S. Miller.

roxy-16-methylpregna-1,4-dione], the arachidonic acid analog 5,8,11,14-eicosatetraynoic acid (ETYA), one of the cyclooxygenase inhibitors ibuprofen [α-methyl-4-(2-methylpropyl)benzeneacetic acid], indomethacin [1-(p-chlorobenzoyl)-5-methoxy-2-methyl-3-indolyl-acetic acid], naproxen [D-2-(6-methoxy-2-naphthyl)propionic acid], piroxicam [3,4-dihydro-2-methyl-4-oxo-N-2-pyridyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide], the dual cyclooxygenase and lipoxygenase inhibitor phenidone [1-phenyl-3-pryazolidinone] or the 5- and 12-lipoxygenase inhibitor esculetin [6,7-dihydroxycoumarin] (all inhibitors from BioMol, Plymouth Meeting, PA). In some experiments, larvae were also injected with 20:4n-6 [5,8,11,14-eicosatetraenoic acid], or 16:0 [hexadecanoic acid] (Sigma Chemical Co., St Louis, MO). Control larvae were injected with nutrient broth, or 95% ethanol. All injections of pharmaceuticals were in a standard volume of 2 μl. The pharmaceutical products were injected at dosages of 4 μg per larva, except in the dose–response experiments, and the fatty acids were injected at dosages of 50 μg per larva. Larvae were surface sterilized by swabbing their surfaces with 95% ethanol. Insects were injected with a standard bacterial dosage of 10^7 cfu/infection (Stanley-Samuelson et al., 1991; Miller et al., 1994). Bacteria were injected in 10 μl aliquots, using a 26 gauge 0.5" needle attached to a 50 μl syringe (Hamilton, Reno, NV). Drugs and control substances were injected in 2 μl aliquots into the opposite side of the larvae using a 10 μl Hamilton 701 syringe (Hamilton, Reno, NV)(6). Nodulation (Fig. 1) was assessed at selected time PI. Larvae were anesthetized by chilling on ice, then the hemocoels were exposed. Melanized, dark nodules were counted under a stereomicroscope at 60×. The nodules were distinct, and direct counting accurately reflected the nodulation response (Miller et al., 1994) After the initial counting, the alimentary canal was excised. Nodules in the previously unexposed areas and remaining internal tissues were then counted.

Control experiments

Because the insects used in these experiments were not reared under semi-sterile conditions, it was expected that untreated insects would have a few nodules. To determine background nodulation, a total of 25 larvae were taken from culture, at various times in this project, anesthetized on ice for 10 min and nodulation was assessed. To assess the influence of injection wounds on nodule formation, 10 larvae were treated by intrahemocoelic infection of a standard volume of ethanol, the drug vehicle. Nodulation was assessed 18 h later. To assess the effect of dexamethasone on nodulation in uninfected larvae, 4 μg of dexamethasone in 2 μl ethanol was infected into 10 larvae. Nodulation was assessed 18 h later. To control for the possibility that nutrient broth could stimulate nodulation, 15 larvae were similarly treated by intrahemocoelic injection of a standard volume (10 μl) of nutrient broth. Nodulation was assessed 18 h later.

Time–course of nodulation: influence of dexamethasone

Individuals in two groups of larvae were treated with 2 μl of ethanol or with 4 μg of dexamethasone in 2 μl of ethanol, and then injected with heat killed bacteria as described. At 1, 2, 4, 6, 18 and 24 h PI, sub-groups of control and experimental larvae were anesthetized, and nodulation was assessed.

Dose–response relationship for dexamethasone

Individuals in six groups of larvae were treated with 2 μl of ethanol, or with 4×10^{-6}, 4×10^{-4}, 4×10^{-2}, 4×10^{-1}, or 4 μg of dexamethasone in 2 μl of ethanol, and then injected with heat killed bacteria. At 18 h PI, the larvae were anesthetized, and nodulation was assessed.

Fatty acid rescue experiments

Individuals in two groups of larvae were injected with either 2 μl of ethanol or 4 μg of dexamethasone in 2 μl of ethanol, and then injected with heat killed bacteria as described, immediately PI, the dexamethasone-treated larvae were divided into three sub-groups. Individuals in each of two sub-groups were treated with 50 μg infections of either 20:4n-6, or 16:0 in 2 μl of ethanol. Individuals in the third sub-group were similarly treated with 2 μl ethanol to control for the effects of the extra injection on nodulation. At 18 h PI, the larvae were anesthetized and nodulation was assessed.

Influence of other eicosanoid biosynthesis inhibitors on nodulation

Individuals in groups of test larvae were injected with either (1) one of the cyclooxygenase inhibitors indomethacin, naproxen, ibuprofen, or piroxicam, (2) the dual cyclooxygenase and lipoxygenase inhibitor phenidone, (3) the lipoxygenase inhibitor esculetin, or (4) the arachidonic acid analog ETYA, all in 2 μl of ethanol. Control larvae were injected with 2 μl of ethanol. Three to 10 min after injection of the inhibitors or ethanol, the larvae were injected with heat killed bacteria as described. At 18 h PI, the larvae were anesthetized and nodulation was assessed.

Determination of phospholipid fatty acid compositions

We determined the fatty acid compositions of phospholipids prepared from five tissues and one body segment: midgut, hindgut, Malpighian tubules, cuticle, fat body and head. Individual insects were anesthetized by chilling, then a single tissue was prepared from each insect. A total of 30 insects were thus used to prepare 5 replicate analyses of each of the six tissues. Total lipids were extracted and phospholipids were isolated as described in Howard et al. (1992). The samples were homogenized in 2 ml of chloroform:methanol (2:1, v/v), amended with 50 μl of 2% butylated hydroxytoluene to minimize autoxidation of polyunsaturated fatty acids. Total lipid extracts were applied to thin layer chromatography plates (20×20 cm, 250 μ Silica Gel G; Sigma Chemical Co., St Louis, MO). The plates were developed

in petroleum ether:diethyl ether:acetic acid (80:20:1, v/v). The phospholipids remain at the origin in this chromatographic system. The silica gel band containing the phospholipids were scraped into 15 ml screw cap reaction tubes, and fatty acid methyl esters were formed by refluxing in acidified methanol for 90 min. Fatty acid ethyl esters were extracted from the reaction tubes in hexane, concentrated and analyzed by gas chromatograph and gas chromatography-mass spectrometry.

The fatty acid methyl esters were chromatographed on a Hewlett-Packard 5890 gas chromatograph equipped with a Supelco Wax 10 capillary column (30 m × 0.25 mm, Supelco Inc., Bellefonte, PA), a flame ionization detector and a Hewlett-Packard 3390A recording integrator. The analyses were conducted by temperature programming at 2°C/min from 150 to 240°C, using helium as carrier gas at 0.6 ml/min. Individual components were tentatively identified by comparing retention times of the components to retention times of authentic standards (Sigma Chemical Co.).

Fatty acid identifications were confirmed by gas chromatography-mass spectrometry. The fatty acid methyl esters were analyzed on a Hewlett-Packard 5890 gas chromatograph interfaced with a Hewlett-Packard 5971 electron impact mass selective detector operated at 70 eV. Separations were performed on a Supelcowax 10 capillary column (30 m × 0.25 mm; Supelco, Inc.) programmed at 1°C per min from 170 to 220°C. Chromatographic conditions included a 45-s splitless injection, a 2-min initial hold period, and the use of ultrapure helium as the carrier gas at 1 ml/min. Retention times and total ion mass spectra of fatty acid methyl esters were compared with authentic standards from Sigma Chemical or by comparison to published electron impact mass spectra (McCloskey, 1970; Ryhage and Stenhagen, 1963).

Eicosanoid biosynthesis experiments

We investigated prostaglandin biosynthesis by microsomal enriched preparations of fat body from *Zophobas* larvae following protocols that were established for fat body from *M. sexta* (Stanley-Samuelson and Ogg, 1994). Briefly, fat body was removed from chilled insects, then homogenized in a ground glass homogenizer. The homogenates were then sonicated for 10 s at 40 W using a VibraCell sonicator (VibraCell, Danbury, CT). This preparation was centrifuged for 10 min at 200 g, and the supernatant was centrifuged for another 10 min at 11,500 g, both steps at 4°C. The 11,500 g supernatants are microsomal-enriched preparations that were used in all experiments. Protein concentration in these preparations were determined against bovine serum albumin using the bicinchoninic acid reagent (BCA; Pierce, Rockford, IL). Standard curves and samples were read on a BioTek microtitre plate reader at 565 nm.

Radioactive 20:4n-6 (5,6,8,9,11,12,14,15-^{3}H-20:4n-6, 60–100 Ci/mmol) was purchased from DuPont. The incubation buffer was 0.05 M KH_2PO_4, pH 8.0, amended with co-factor cocktail (2.4 mM reduced glutathione, 0.25 mM hydroquinone and 25 μg hemoglobin). 2.5 μCi

of labeled 20:4n-6, was dispensed into reaction tubes and the solvent was evaporated. The reactions were carried out in 1.0 ml total volume. The experiments were preceded by a 3 min pre-incubation with all reaction components except the protein source. After the appropriate incubation period, the reactions were stopped by addition of 500 μl of 0.1 N HCl. Products were extracted from the acidified reaction mixture three times in ethyl acetate. The combined extracts, containing prostaglandins, and possibly lipoxygenase products, were evaporated under N_2. A mixture of appropriate standards was added to each sample, then samples were applied to thin layer chromatography plates (Sigma Chemical Co.). the plates were developed and fractions observed as described (Stanley-Samuelson and Ogg, 1994). Bands corresponding to selected authentic eicosanoid standards and to free fatty acids were transferred to 24-well microtitre plates, and 750 μl of counting cocktail (Microscint-20 Packard Instrument Co, Meridian, CT) was added to each well. The radioactivity in each sample was estimated by liquid scintillation counting on a Top Count Microplate Scintillation Counter (Packard Instrument Co.) at 40% efficiency for tritium. In negative control experiments, microsomal-enriched preparations were heated in boiling water for 10 min before the experiments, and processed as described. These controls were conducted in parallel with every experiment. Experimental values were corrected according to these controls (Stanley-Samuelson and Ogg, 1994).

Statistical analyses and voucher specimens

Significant treatment effects were confirmed for all nodulation experiments by one-way and two-way analysis of variance with $P \leq 0.05$ using the personal computer version 6 of Stratgraphics (STSC, Rockville, MD). Where appropriate, significant differences among treatment means were determined by protected Least Significant Differences (LSD). Voucher specimens (as adults) have been deposited in the research collection of the Department of Entomology, Kansas State University, Manhattan.

RESULTS

Control experiments

The results of the control experiments are displayed in Table 1. To examine the effects of injection of nodule

TABLE 1. Outcomes of background control experiments.The values are mean number of nodules per larvae (1 SEM). Nodulation was assessed at 18 h PI

Treatment	Nodulation	Number of larvae
No treatment	1.5 (0.6)	25
Vehicle only	1.8 (0.6)	10
Dexamethasone only	0.8 (0.4)	10
Nutrient broth only	1.9 (0.5)	15
Live bacteria	37.7 (2.1)	37
Heat killed bacteria	41.8 (5.7)	13

formation, 10 μl doses of ethanol, the standard carrier vehicle for all pharmaceuticals, were injected into larvae. A mean of 1.8 (SE=0.6; n=10) nodules were observed. Dexamethasone treatments caused about 0.8 nodule per larva. Treatments with nutrient broth resulted in about 2 nodules per larva. Injections of standard dosages of live and heat killed *S. marcescens* resulted in about 40 nodules per larva.

Time course of nodulation

Figure 2 displays the time course of visible nodulation in two groups of larvae. Dexamethasone-treated larvae formed about 1–2 nodules at 1 h PI, which increased to about 17 at 6 h PI. Nodulation did not increase during the next 18 h PI. The ethanol-treated control larvae produced significantly more nodules at each time point, from 8 nodules at 1 h PI, to 24 at 6 h PI, and to 43 nodules per larva at 24 h PI (LSD, $P \leq 0.05$). Apparent maximum nodulation obtained at 18 h PI, and in all subsequent experiments nodulation was assessed at 18 h PI.

Dose–response curve for dexamethasone

The relationship between dexamethasone dosage and number of nodules is shown in Fig. 3. Increased dexamethasone dosages were associated with decreased nodulation in response to bacterial infections, from about 40 to about 20 nodules per larva.

Fatty acid rescue experiments

As we have just seen, dexamethasone treatments inhibited the ability of *Zophobas* larvae to form nodules in response to bacterial infections. On the model that dexamethasone inhibits eicosanoid biosynthesis through its effects on PLA_2, we reasoned that injecting eicos-

anoid-precursor fatty acids into dexamethasone-treated heat killed bacteria injected larvae should reverse the effects of dexamethasone on nodulation. Figure 4 shows that the 20:4n-6 treatments reversed the effects of dexamethasone on nodulation. Ethanol-injected control larvae produced about 40 nodules per larva, and dexamethasone treated larvae produced about 23 nodules per larva, as expected from earlier experiments. The 20:4n-6 treated larvae produced about 40 nodules per larva, on a par with the control animals. Dexamethasone treated larvae subsequently injected with ethanol produced about 25 nodules, indicating that a second injection did not exert a significant effect on nodulation. The rescue effects were specific to eicosanoid-precursors, because 16:0, which cannot be desaturated to form C20 polyunsaturated fatty acids, did not restore nodulation to control levels (LSD, $P<0.05$).

Influence of other eicosanoid biosynthesis inhibitors on nodulation

To dissect the possible roles of cyclooxygenase and lipoxygenase pathways in nodulation, *Zophobas* larvae were treated with standard dosages of four cyclooxygenase inhibitors, one dual cyclooxygenase and lipoxygenase inhibitor, one 5-and 12-lipoxygenase inhibitor or with the arachidonate analog ETYA, and then injected with heat killed bacteria. Figure 5 shows that compared to control larvae, all test larvae exhibited significantly reduced nodulation in response to bacterial injections LSD, $P<0.05$). There were no significant differences among the effects of individual inhibitors on nodulation except for naproxen, which was somewhat less effective than the others.
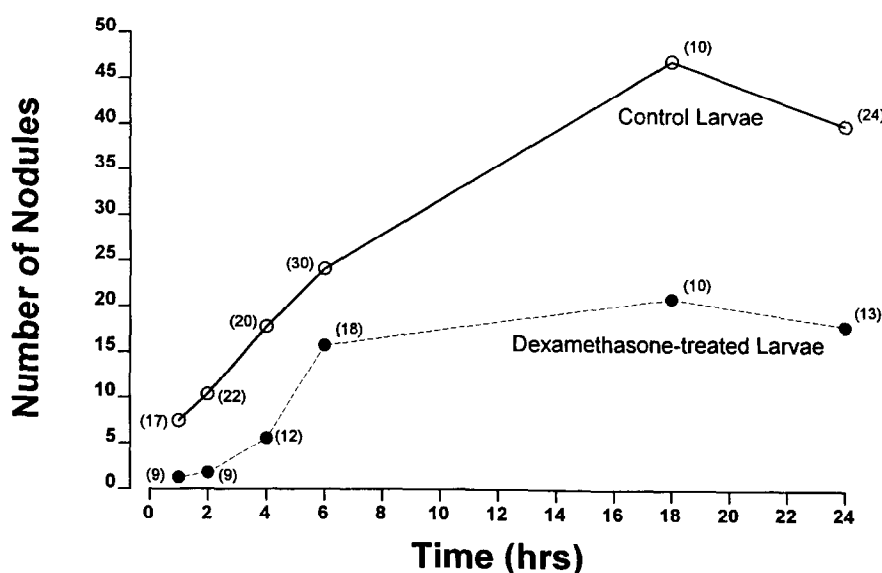

FIGURE 2. Time–course for *Z. atratus* nodulation in response to intrahemocoelic injections with heat killed cells of the pathogenic bacterium *S. marcescens*. Test larvae (lower line) were first injected with dexamethasone, and control larvae (upper line) were first injected with ethanol. Within 3–10 min, both groups of insects were anesthetized on ice, and nodulation was assessed. Each point indicates the mean number of nodules found in each larva (number of larvae), and the error bars represent 1 SEM.
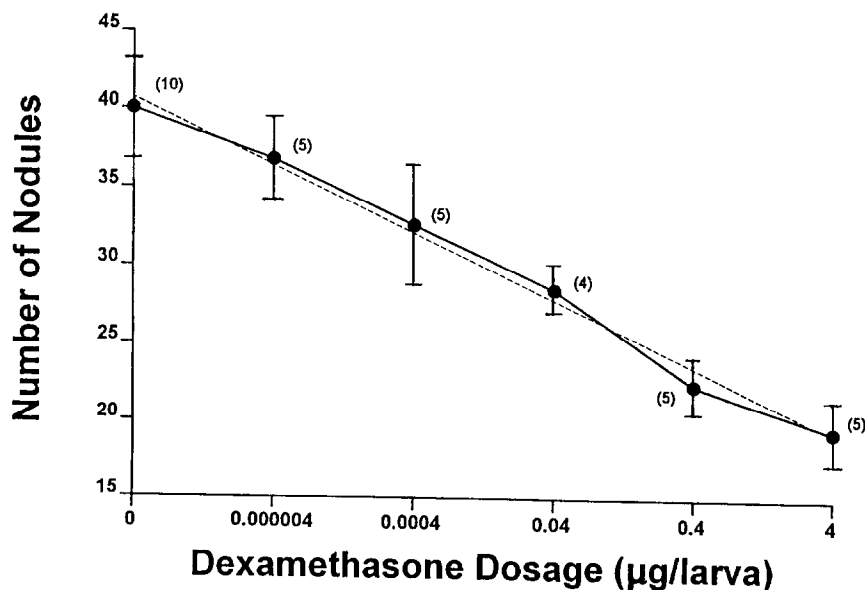
FIGURE 3. Dose-response for dexamethasone: nodule formation. *Z. atratus* larvae were first injected with the indicated dosages of dexamethsone, and then intrahemocoelically injected with heat killed bacteria. After 18 h incubation, the insects were anesthetized on ice, and nodulation was assessed. Each point indicates the mean number of nodules found in each larva (number of larvae), and the error bars represent 1 SEM.

Fatty acid compositions

The fatty acid compositions of phospholipids prepared from six larval *Zophobas* tissues are presented in Table 2. As expected from the general background of insect fatty acid biochemistry, 16:0, 18:1 and 18:2n-6 were the quantitatively major components of all tissues. Several odd-carbon number fatty acids were also detected, including 15:0, 17:0, 17:1 and 19:0. Eicosanoid precursor polyunsaturated fatty acids, 20:3n-6, 20:4n-6 and 20:5n-3, were present in trace levels in all tissues. It is worth

noting that these components were detectable by mass spectrometry, but were not readily discernible by flame ionization gas chromatography. As Table 2 indicates, there were moderate differences in overall fatty acid profiles among the various tissue sources.

Eicosanoid biosynthesis

Table 3 shows that the fat body preparation from *Zophobas* was competent to biosynthesize eicosanoids. Four cyclooxygenase products, PGA_2, PGE_2, PGD_2 and
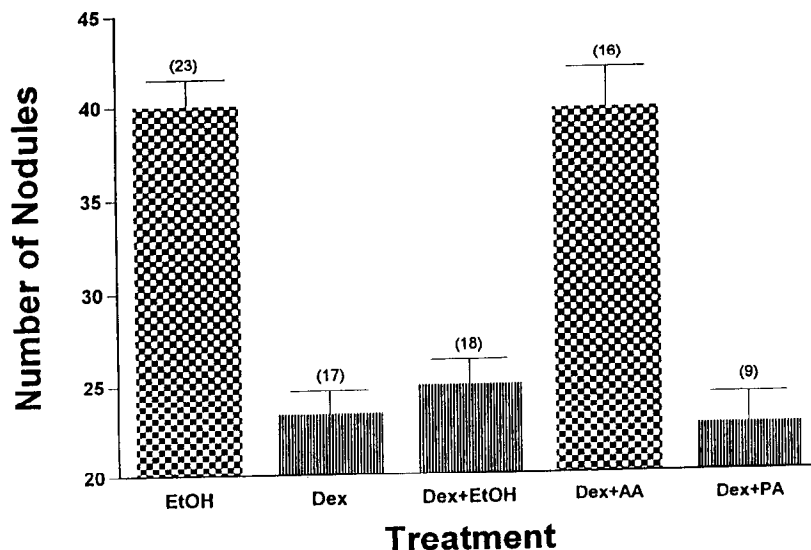

FIGURE 4. Eicosanoid-precursor fatty acids reversed the dexamethasone effect on nodulation. *Z. atratus* larvae were treated with ethanol (ETOH), or dexamethasone (DEX), then intrahemocoelically injected with heat killed bacteria. Immediately PI, test larvae were treated with 50 μg of either 16.0 (DEX + PA), or 20:4n-6 (DEX + AA). Control larvae were treated with ethanol (DEX+ETOH). At 18 h PI, the larvae were anesthetized and nodulation was assessed. The height of histogram bars represent the mean number of modules found in each larvae, and the error bars represent 1 SEM. The number of larvae in each category is indicated above the error bars. Histogram bars with the same fill pattern are not significantly different from each other (LSD, $P<0.05$).
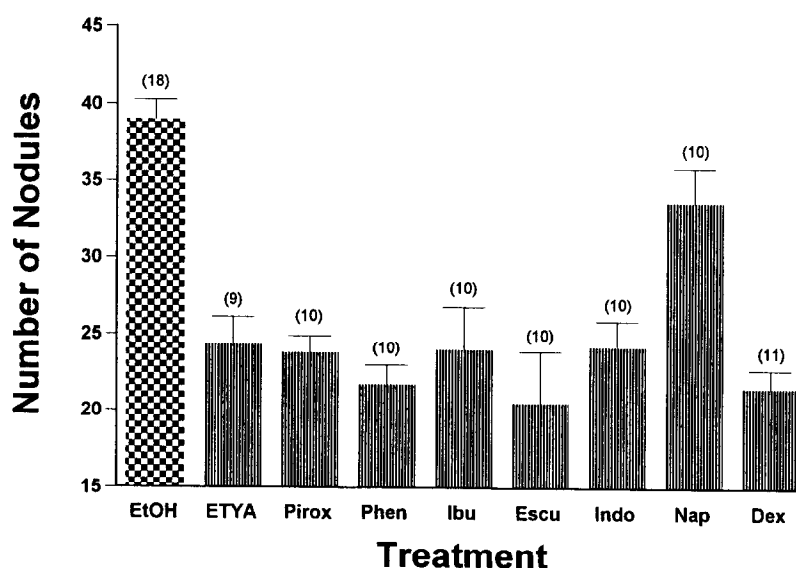
FIGURE 5. Effect of treating *Z. atratus* larvae with individual eicosanoid biosynthesis inhibitors on nodule formation in response to intrahemocoelic injections with heat killed bacteria. Test larvae were first injected with 4 μg of the indicated inhibitor. Control larvae were first injected with ethanol (ETOH). Test and control larvae were then intrahemocoelically injected with heat killed bacteria. At 18 h PI, larva were anesthetized and nodulation was assessed. The height of histogram bars represent the mean number of nodules found in each larva, and the error bars represent 1 SE of the mean. The number of larvae in each category is indicated above the error bars. Histogram bars with the same fill pattern are not significantly different from each other (LSD, $P<0.05$).

$PGF_{2\alpha}$ and a lipoxygenase product that co-chromatographed with 15-hydroxyeicosa-tetraenoic acid, were detected. As in the *Manduca* fat body, PGA_2 was the major product (Stanley-Samuelson and Ogg, 1994).

DISCUSSION

The data presented in this paper support the hypothesis that eicosanoids mediate the nodulation response to bacterial infections in the beetle *Z. atratus*. This finding is

TABLE 2. Percent composition of phospholipid fatty acids from six tissues of *Zophobas atratus* larvae ($N=5$). Fatty acids compositions as percentages of total fatty acids, of phospholipids prepared from 5 tissues and heads of *Z. atratus* larvae. Values are mean percentages (1 SEM), $N=5$ separate analyses

Fatty acid	Midgut	Malpighian tubule	Cuticle	Fat body	Head	Hindgut
14:0	0.88 (0.18)	5.38 (0.54)	1.75 (0.11)	1.57 (0.25)	1.63 (0.38)	1.66 (0.30)
15:0	0.19 (0.02)	0.65 (0.13)	0.10 (0.02)	0.23 (0.07)	0.23 (0.03)	0.27 (0.03)
16:0	11.52 (0.26)	9.29 (1.83)	13.02 (0.39)	20.53 (1.23)	16.21 (2.31)	13.32 (0.64)
16:1A	0.44 (0.04)	1.16 (0.17)	0.77 (0.27)	1.27 (0.24)	0.96 (0.29)	0.46 (0.04)
16:1B	0.20 (0.01)	0.19 (0.04)	0.11 (0.03)	0.41 (0.05)	0.30 (0.11)	0.14 (0.01)
16:2	0.12 (0.04)	0.25 (0.13)	0.05 (0.02)	0.52 (0.23)	0.33 (0.21)	0.21 (0.03)
17:0	0.96 (0.05)	0.26 (0.16)	1.66 (1.32)	0.40 (0.06)	0.57 (0.09)	0.62 (0.05)
17:1	0.22 (0.06)	0.01 (0.01)	0.17 (0.06)	0.06 (0.02)	0.16 (0.09)	0.39 (0.06)
18:0	12.00 (0.72)	9.65 (0.61)	5.87 (0.22)	8.00 (0.49)	6.84 (0.61)	10.40 (0.67)
18:1	17.17 (0.85)	19.67 (0.96)	17.88 (0.41)	20.27 (2.25)	21.35 (2.71)	24.64 (1.46)
18:2	51.78 (2.48)	53.24 (2.20)	57.82 (1.32)	45.35 (3.42)	50.35 (4.99)	46.14 (1.02)
19:0	0.37 (0.05)	<0.01	<0.01	0.03 (0.01)	0.01 (0.01)	0.07 (0.03)
18:3	0.95 (0.19)	0.24 (0.24)	0.64 (0.18)	0.66 (0.05)	0.99 (0.16)	0.37 (0.05)
20.0	2.95 (2.53)	<0.01	0.12 (0.05)	0.64 (0.31)	0.01 (0.01)	0.73 (0.02)
20:1	0.01 (0.01)	<0.01	<0.01	<0.01	<0.01	<0.01
20:2	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
20:3n-6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
20:4n-6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
20:3n-3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
20:5n-3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
22:0	0.21 (0.03)	<0.01	0.04 (0.04)	0.07 (0.07)	0.05 (0.05)	0.57 (0.07)

TABLE 3. Eicosanoid biosynthesis by microsomal enriched preparations of fat body from larvae of the beetle, *Z. atratus*. Following standard protocol, 2 mg of microsomal-enriched protein were incubated in the presence of radioactive 20:4n-6 for 2 min at 32°C. Eicosanoids were extracted from the reaction mixtures, and separated by thin-layer chromatography. Radioactivity in fractions that co-chromatographed with authentic standards was assessed by liquid scintillation counting. Values represent eicosanoid biosynthesis as pmol/mg/h, ± 1 SE, $N=12$ separate experiments

Eicosanoid product	Biosynthesis rate	Standard error
PGA$_2$	0.28	0.06
PGD$_2$	0.09	0.03
PGE$_2$	0.10	0.04
PGF$_{2\alpha}$	0.05	0.02
HETE[a]	0.70	0.19

[a]HETE=hydroxyeicosatetraenoic acid.

congruent with our original findings with the tobacco hornworm, *M. sexta* (Miller *et al.*, 1994). Our conclusions are supported by the following points. First, the dexamethasone effect on nodulation began within the first hr PI, and increased relative to control animals throughout the time course of the experiments. Second, the dexamethasone effects on nodule formation were expressed in a dose-dependent manner. Third, the influence of dexamethasone was reversed by treating the larvae with free eicosanoid-precursor fatty acids. Fourth, inhibition of eicosanoid biosynthesis with specific inhibitors of PLA$_2$, cyclooxygenase, and lipoxygenase significantly reduced nodulation in infected larvae. Fifth, eicosanoid-precursor polyunsaturated fatty acids were detected in all the beetle larvae tissues examined. Finally, microsomal-enriched fat body preparations expressed cyclooxygenase and lipoxygenase activities.

Nodules are easily visible in both tobacco hornworms and *Z. atratus* because they are darkly melanized. In our work with the tobacco hornworms it was not immediately clear whether eicosanoids mediated aggregation of hemocytes, which leads to nodulation, or if eicosanoids mediated the melanization reactions, which would make nodules easily visible. We dissected these possibilities in *M. sexta* by observing the effects of dexamethasone on formation of microaggregates within the first few minutes PI. We found that dexamethasone significantly reduced the formation of microaggregates in those larvae, from which we proposed that eicosanoids mediate the early and later stages of nodulation (Miller *et al.*, 1994). Due to technical difficulties in working with the very small amount of hemolymph in *Zophobas*, we were unable to directly observe the influence of dexamethasone on early microaggregate formation. Nevertheless, the pronounced effects of dexamethasone and other eicosanoid biosynthesis inhibitors allows us to propose that eicosanoids mediate nodulation in *Zophobas* in the same manner as suggested for *Manduca*. While not ruling out the possible influence of eicosanoids on melanization reactions, we propose that eicosanoids mediate the nodulation response to bacterial infections in *Manduca* and in *Zophobas*.

Because the melanization responses to injection wounds can produce the appearance of nodulation, we conducted experiments designed to control for background nodulation (Table 1). Only a few nodules were seen in insects taken directly from our culture. Because these insects were not reared and maintained in a semi-sterile culture, a few nodules are expected. In our experience with the semi-sterile *Manduca* culture, we observed substantial levels of nodulation only in hornworms that had been adventiously contaminated with fungal infections. Injections of the drug vehicle, of dexamethasone, and of sterile nutrient broth uniformly resulted in negligible background nodulation (Table 1). These data indicate that the high level of nodulation we observed was a specific response to bacterial challenge, and not a reflection of the insects' background health.

After injection of about the same number of bacterial cells, tobacco hornworms typically form about 3 times as many nodules as we observed in *Zophobas* larvae. This may be due to differences in the total hemocyte population in the two species. Both species, as well as many other insects, appear to have approximately similar concentrations of circulating hemocytes (*ca* 2–6 10^6/ml) but the total hemolymph volume of fifth instar *Manduca* larvae is several times the hemolymph volume of *Zophobas* larvae. These differences in volume translate into substantial differences in actual numbers of circulating hemocytes available to form nodules. Although the point remains to be investigated, hemolymph volume and its respective hemocyte population may set an upper limit on the capacity for nodulation in insects.

The approximate linear relationship between logarithmically increasing dexamethasone dosages and decreasing nodulation, and the reversal of the dexamethasone effects on nodulation with eicosanoid-precursor fatty acids (Figs 3 and 4) powerfully argue that eicosanoids mediate insect nodulation responses to bacterial infections. The dexamethasone effects on eicosanoid biosynthesis are thought to act at the level of PLA$_2$, which catalyzes hydrolysis of fatty acids from cellular phospholipids. If dexamethasone exerts its effects by inhibiting PLA$_2$ in *Zophobas*, then subsequent treatments with eicosanoid-precursors should reverse the dexamethasone effects. Figure 4 shows that, indeed, 20:4n-6 restored nodulation in dexamethasone-treated infected larvae. An apparent restoration of nodulation could have resulted from non-specific effects, such as additional handling, the extra injection required to administer the fatty acids after infecting the larvae, or a pharmacological effect of the fatty acids. However, handling and injecting 2 μl of ethanol into dexamethasone-treated infected larvae did not increase nodulation. Similarly, injecting 16:0, which cannot lead to eicosanoid biosynthesis, only slightly (and non-significantly) increased nodulation, and did not restore nodulation to the level seen in control larvae or eicosanoid-precursor treated larvae. These experiments support the idea that eicosanoids mediate the nodulation response to bacterial infections in insects.

A crucial point in all of these discussions is that the

insect must have the necessary precursor fatty acids and necessary enzymes for biosynthesis of eicosanoids. The phospholipid fatty acid profiles of Z. atratus samples we examined (Table 2) all indicate small, but measurable quantities of these fatty acids. The low levels found of these acids are in agreement with most reports of terrestrial arthropods (Stanley-Samuelson and Dadd, 1983; Stanley-Samuelson et al., 1988), including Tenebrio molitor (Howard and Stanley-Samuelson, 1990), a species with many phylogenetic and ecological similarities to Z. atratus. Because eicosanoids function in only trace amounts (Stanley-Samuelson, 1994a) there is no physiological demand for an organism to produce or store large quantities of precursor fatty acids. In those animals where substantial quantities of these fatty acids are found (Stanley-Samuelson et al., 1988), it is likely that they serve additional functions besides serving as substrate for eicosanoid biosynthesis. As Table 3 indicates, Z. atratus fat body is clearly competent to produce a variety of eicosanoid products at rates comparable to that found in other insect studies (Stanley-Samuelson and Ogg, 1994).

We found that a wide range of eicosanoid biosynthesis inhibitors effectively reduced nodulation in infected Z. atratus larvae (Fig. 5). Nodulation is a complex, multistep process (Gupta, 1991) which typically begins with recognition of non-self cells or tissues. The point to offer is that various eicosanoids, including both cyclooxygenase and lipoxygenase products, may be involved in a number of specific cellular events in the complete nodulation process. Inhibition of any one or more of the eicosanoid-mediated steps may curtail an overall nodulation process. If this is so, it is not surprising that inhibition of either of the major eicosanoid-biosynthetic pathways would similarly inhibit nodulation.

We recognize that a major difficulty with using pharmaceutical inhibitors to probe the possible roles of eicosanoids in physiological systems in invertebrates is the possibility of non-specific and non-physiological effects (Stanley-Samuelson, 1994b). We have addressed this issue by arguing from cases in which the inhibitors we used have been shown to have similar pharmacological actions in invertebrates and in mammals (Stanley-Samuelson et al., 1991; Miller et al., 1994). Moreover, we recently obtained biochemical evidence that very low levels of naproxen and indomethacin (0.001 μM) strongly inhibited cyclooxygenase activity in fat body and hemocyte preparations from tobacco hornworms (Stanley-Samuelson and Ogg, 1994). Use of inhibitors to probe these pathways also rests upon the assumption that the inhibitory compounds reach the target tissues in vivo. Preliminary data indicate that one inhibitor, indomethacin, is rapidly accumulated from hemolymph circulation by tobacco hornworm tissues, including hemocytes and fat body (Miller and Stanley-Samuelson, unpublished). Considerably more work on the pharmacology and the quantitative influence of putative eicosanoid biosynthesis inhibitors on in vivo eicosanoid titres in insects is required before these compounds can be completely accepted as unequivocal probes for assessing

the influence of eicosanoids in insect physiology (Stanley-Samuelson, 1994b). The evidence to date, however, is clearly sufficient to justify our assertion that the compounds used in our experiments expressed their effects through inhibition of eicosanoid biosynthesis.

Cyclooxygenase and lipoxygenase products are crucial mediators of many aspects of mammalian physiology, including cellular host defense actions. For example, cyclooxygenase products have profound effects on macrophage locomotion, cell shape changes and phagocytosis, and lipoxygenase products mediate chemotaxis, chemokinesis and adherence responses of neutrophils (Levine, 1988; Ninnemann, 1988). In mammals hemocytic immune responses are mediated by a number of independent factors, including cytokines, biogenic amines, platelet activating factors and eicosanoids. Although we have only scant data on the point, regulation of invertebrate hemocyte actions will almost certainly prove to be similarly complex. For example, Baines et al. (1992) suggest that octopamine and 5-hydroxytryptamine enhance hemocytic defense reactions in the cockroach Periplaneta americana. Again, to the extent that the cockroach data serves as a general model of invertebrate immunity, we can expect to learn that biogenic amines mediate cellular defenses in most invertebrates. We also note that inhibiting eicosanoid biosynthesis in either Manduca or Zophobas did not completely block nodule formation. It would appear that other factors are responsible for the nodulation that occurred in the presence of eicosanoid biosynthesis inhibitors. We suggest that many factors, not limited to the eicosanoids and amines just discussed, are involved in regulating invertebrate hemocytic immune responses. Coupled with the background of mammalian studies, our findings with insects allow us to propose that eicosanoids mediate cellular defenses in both vertebrates and invertebrates. This idea is also supported by recent findings on blood cells from the crab, Carcinus maenas. These crab cells biosynthesize eicosanoids, including cyclooxygenase and lipoxygenase products (Hampson et al., 1992), which may similarly mediate defense responses.

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